Endothelial cell–lined skeletal muscle ventricles in circulation

Skeletal muscle ventricles were constructed from the latissimus dorsi in six dogs by wrapping the muscle around a polypropylene mandrel. Jugular vein endothelial cells were harvested enzymatically and grown in tissue culture. After 3 weeks of vascular delay and 4 weeks of electrical conditioning, five skeletal muscle ventricles were seeded with 5 to 8 × 10^6 autologous endothelial cells by percutaneous injection of a cellular suspension into the lumen of the skeletal muscle ventricle; one skeletal muscle ventricle was injected with culture medium alone as an unseeded control. The autologous endothelial cells were all prelabeled with a lipid-bound cellular marker, PKH-26. After an additional 4 weeks of electrical conditioning, the mandrels were removed and the skeletal muscle ventricles were connected to the descending thoracic aorta and activated to contract during cardiac diastole at a 1:2 ratio with the heart. After 3 hours of continuous pumping, mean diastolic pressure was increased by 35% (58 ± 7 versus 78 ± 6 mm Hg, $p < 0.05$). At this time, the skeletal muscle ventricles were excised for histologic examination. Sections stained with hematoxylin and eosin revealed a continuous cellular layer lining the skeletal muscle ventricle; no cells were present on the lumen of the control skeletal muscle ventricle. All seeded skeletal muscle ventricles exhibited fluorescence as a result of the PKH-26 cellular marker. Immunofluorescent staining with antibodies to von Willebrand factor and ultrastructural analysis with an electron microscope confirmed the endothelial character of these cells lining the lumen of the skeletal muscle ventricles. The ability to create endothelial cell–lined muscular pumping chambers holds important implications for the resolution of thrombotic events in cardiac assist devices as well as toward the clinical application of skeletal muscle ventricles. (J THORAC CARDIOVASC SURG 1995;109:66-73)

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Although several investigators have wrapped skeletal muscle directly around the heart for cardiac assist, we have worked toward developing separate pumping chambers from skeletal muscle, termed *skeletal muscle ventricles,* or *SMVs.* One of the difficulties encountered during long-term studies of SMVs in circulation has been the development of thrombus inside the SMV.[1-3] The two biggest factors involved in SMV thrombosis are the flow characteristics and the blood-surface interactions within the SMV cavity. An endothelial monolayer might decrease the likelihood of thrombosis by providing nature's own antithrombotic surface. In previous studies, we[4-6] have demonstrated the feasibility of seeding the surfaces of SMVs with endothelial cells. The purpose of this study was to determine whether autogenously seeded, endothelial cell–lined SMVs could retain this lining while actively pumping blood in the systemic circulation.

Materials and methods

SMV construction. SMVs were constructed from the latissimus dorsi muscle in six dogs as previously described.[7] All animals were operated on in accordance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH publication No. 85-23, revised 1985). In brief, the latissimus dorsi muscle was mobilized except for its neurovascular pedicle and humeral insertion. A nerve lead was placed around the thoracodorsal nerve and connected to a neurostimulator (Itrel, Medtronic Inc., Minneapolis, Minn.). The muscle was wrapped approximately two times around a synthetic, polypropylene mandrel. At the same time, the jugular vein was excised and placed in 50 ml of a phosphate-buffered saline solution with 4 to 5 ml of oxygenated blood and 2000 units of sodium heparin at 37° C for later harvesting. The neurostimulator was buried under the left rectus muscle, all wounds were closed in layers, and the animals recovered.

Endothelial cell culture. Autologous endothelial cells were harvested from the jugular vein with 0.1% collagenase and gentle scraping. Contaminants were removed by flow-cytometric sorting with the endothelial cell–specific marker 1,1′-dioctadecyl-3,3,3′,3′ tetramethylnidocarbocyanine-perchlorate (diI)-acetylated low-density lipoprotein.[8] The purified, autologous endothelial cell population was frozen in 10% dimethyl sulfoxide at −80° C and stored at −196° C. After 4 weeks, the cells were thawed and reconstituted in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, L-glutamine (2 mmol/L), and antibiotics. The cells were maintained in a 5% carbon dioxide incubator at 37° C and underwent a total of three to five passages in culture until a total of 5 to 8 × 10^6 cells per animal were available for seeding.

Endothelial cell seeding. While the endothelial cells were being cultured, the animal's SMV underwent a 3-week vascular delay followed by a 4-week period of electrical conditioning at 2 Hz. This regimen has been shown to transform the muscle to a fatigue-resistant state.[9] After this 7-week period, the animals underwent seeding by percutaneous injection of a solution (5 × 10^8 cells per animal) containing each animal's autologous endothelial cells suspended in 5 ml of Dulbecco's modified Eagle's medium. Immediately before seeding, the pure endothelial cell population was prelabeled with a lipid-bound marker, PKH-26. This marker has been previously demonstrated to be a reliable cellular label to track cells in vivo.[10] The cells were then injected into the space adjacent to the mandrel around which the latissimus muscle had been wrapped. Several injection sites were used to help ensure uniformity of seeding. One animal underwent injection of Dulbecco's modified Eagle's medium only as an unseeded control. The neurostimulator was temporarily turned off during the seeding process.

Hemodynamic measurements. One week after seeding, the neurostimulators were reactivated at 2 Hz and conditioning was continued for an additional 3 weeks. At the conclusion of this time (4 weeks from seeding, 11 weeks from construction), the animals were reanesthetized and the chest was opened via a lateral thoracotomy. The mandrel was carefully removed from the SMV and the SMV was anastomosed to the descending thoracic aorta with two polytetrafluoroethylene grafts. The nerve lead was attached to a cardiomyostimulator (Prometheus, Medtronic Inc.) and two myocardial sensing electrodes were placed on the heart. The stimulator was set at a 33 Hz burst frequency at a 1:2 contraction ratio with the heart. The burst delay was set at 40% with a burst duration of 45% of the R-R interval. Aortic and left ventricular pressures were recorded with 5F microtransducer-tipped catheters positioned at the time of the operation. Hemodynamic recordings were taken at the time of connection to the circulation and hourly for 3 hours.

Histologic examination. After 3 hours of continuous pumping in circulation, the animals were killed and the SMVs carefully excised. The SMVs were washed gently in saline solution, and the surface was then fixed in situ with Karnovsky's fixative for 20 minutes at room temperature; the SMVs were subsequently fixed in formalin at 4° C for 24 hours. The luminal surface was divided into three areas: top, middle, and apex (bottom). After initial examination for gross evidence of thrombosis or damage to the luminal surface, at least six random segments, approximately 1 cm^2 each, were excised from the three different portions. All samples were coded and then processed and evaluated in a blinded fashion as to the state of endothelialization of the sections. Two samples from each segment were processed for conventional histologic examination (hematoxylin and eosin staining), and two were analyzed *en face* as whole mounts by fluorescence microscopy with PKH-26 and von Willebrand factor (vWF). The remaining samples were set aside for electron microscopy.

Sections stained with hematoxylin and eosin were analyzed qualitatively for the presence or absence of a contiguous monolayer of cells with a standard light micro-

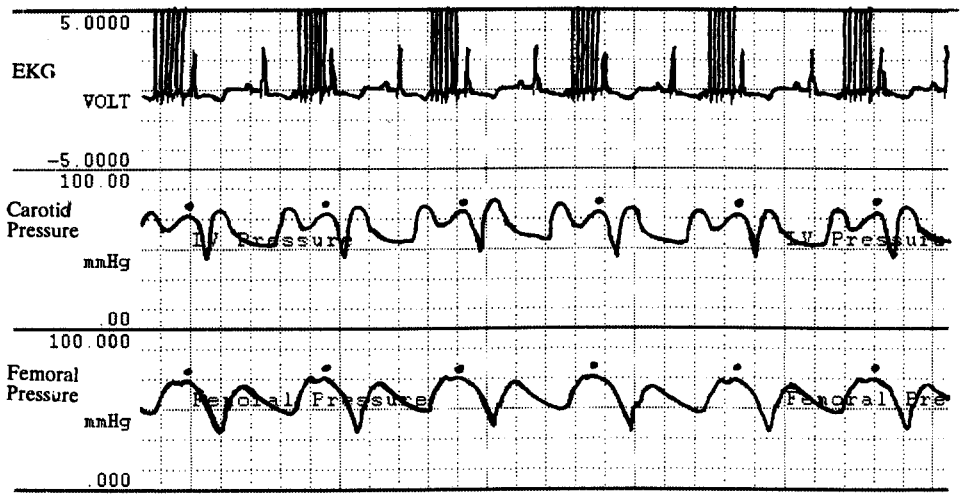

Fig. 1. Representative pressure trace taken from an animal with an endothelialized SMV in circulation for 3 hours. Diastolic augmentation noted by *dots* in carotid and femoral traces. Cardiomyostimulator contracting at a 1:2 ratio with the heart at 33 Hz burst frequency. *EKG,* Electrocardiogram.

Table I. *Hemodynamic assessment of endothelial cell–lined SMV aortic counterpulsation*

	Initial		*One hour*		*Two hours*		*Three hours*	
Parameter	*Off*	*On*	*Off*	*On*	*Off*	*On*	*Off*	*On*
Heart rate (beats/min)	107 ± 8	107 ± 8	107 ± 9	107 ± 9	114 ± 8	114 ± 8	114 ± 7	114 ± 7
BP_{SYS} (mm Hg)	80.3 ± 5.1	76.5 ± 7.6	83.4 ± 7.2	82.9 ± 7.2	82.7 ± 5.7	82.0 ± 5.5	78.2 ± 5.5	76.9 ± 5.7
BP_{DIA} (mm Hg)	58.6 ± 5.1	78.1 ± 5.8*	62.4 ± 7.7	80.8 ± 6.4*	61.6 ± 5.8	80.8 ± 6.4*	58.3 ± 7.1	78.2 ± 6.2*
$BP_{PRESYSTOLE}$	54.3 ± 4.9	45.3 ± 5.5*	58.1 ± 7.9	51.9 ± 8.9	57.2 ± 6.4	48.6 ± 7.6*	53.7 ± 6.3	48.7 ± 7.0
TTI (mm Hg · sec)	16.7 ± 0.9	15.2 ± 1.1*	16.2 ± 0.6	15.2 ± 2.6	15.8 ± 1.1	15.1 ± 1.4	14.2 ± 1.1	13.8 ± 1.3
DPTI (mm Hg · sec)	19.9 ± 1.5	23.2 ± 2.1*	21.0 ± 2.1	25.7 ± 2.0*	19.3 ± 2.0	23.5 ± 2.8*	18.2 ± 1.7	21.8 ± 1.9*
EVR (DPTI/TTI)	1.19 ± 0.06	1.52 ± 0.09*	1.27 ± 0.09	1.71 ± 0.01*	1.24 ± 0.14	1.61 ± 0.55	1.28 ± 0.14	1.67 ± 0.11*
Diastolic augment (%)	NA	33.2 ± 8.3	NA	29.5 ± 10.1	NA	31.2 ± 9.0	NA	34.1 ± 6.0

BP_{SYS}, Peak systolic carotid pressure; BP_{DIA}, mean diastolic carotid pressure; $BP_{PRESYSTOLE}$, minimum diastolic carotid pressure; *TTI,* systolic tension time index; *DPTI,* diastolic tension time index; *EVR,* endocardial viability ratio; *diastolic augment,* [BP_{DIA} (on) − BP_{DIA} (off)]/BP_{DIA}; *NA,* not applicable.
*$p < 0.05$ (analysis of variance), pacer *off* versus *on* ($n = 6$).

scope. Fluorescence staining of the en face blocks was viewed with a low light–level video camera (Hamamatsu Photonics KK, Shizuoka, Japan) and digitized into a TN8500 image analyzer (Noran Instruments, Middleton, Wis.), with the red, rhodamine-fluorescence filter used for PKH-26 fluorescence. Background autofluorescence was determined by using the unseeded, control SMV as a zero reference.

The en face whole mount segments were then stained immunofluorescently with labeled antibodies to vWF. The sections were incubated with a rabbit-derived 1:100 dilution of polyclonal vWF antibody. Bovine serum albumin (0.1%) was used to block nonspecific binding. A 1:1000 dilution antirabbit immunoglobulin G labeled with fluorescein isothiocyanate (FITC) was then used to label the vWF antibody. Sections were viewed with fluorescein excitation and the green, FITC-fluorescent filter was used. Filtered images were digitized with the TN8500 image analyzer as described earlier, again with the autofluorescence of the unseeded SMV used as a control.

From each of these tissue samples (light and fluorescent microscopy), three random areas were photographed and evaluated. With the use of the en face mounts with PKH-26 and vWF staining, the percent surface coverage of endothelial cells over the SMV luminal surface was determined by analyzing the confluency of cellular fluorescence compared with the total area. After evaluations were compiled, the experimental results were correlated with the sample identification, seeding state, and location of sample to determine the total percent cellular coverage for each seeded SMV.

Random sections of one of the seeded SMVs and also from the unseeded SMV were prepared and submitted for examination with an electron microscope.

Results

Hemodynamics. Hemodynamic values for all six animals over the 3-hour period are included in Table I. The SMVs functioned well throughout the

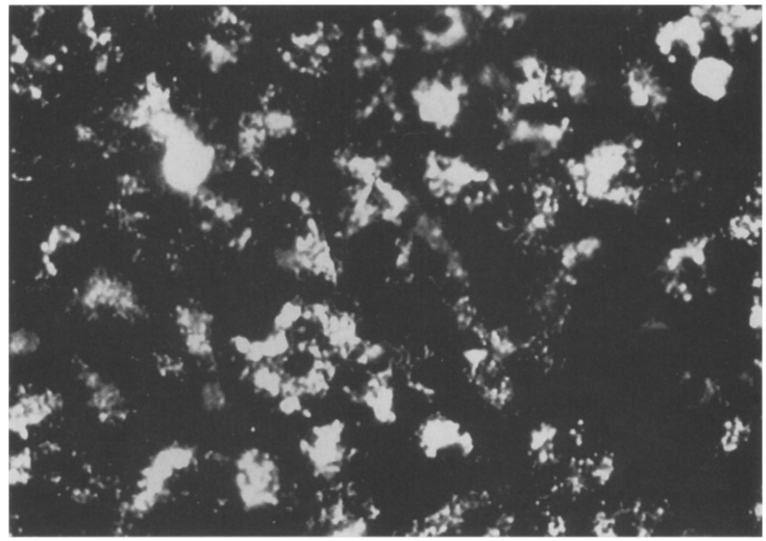

Fig. 2. Photomicrograph of the luminal surface of seeded SMV after incubation with immunofluorescent antibodies to vWF. Presence of vWF staining identifies cells lining the SMV lumen as endothelial in nature. (Original magnification ×650.)

3 hours of continuous pumping; mean diastolic pressure was augmented 35% at the conclusion of the experiment (58 ± 7 versus 78 ± 6 mm Hg, $p < 0.05$). A representative pressure tracing from one animal is shown in Fig. 1.

Light microscopy. Light microscopy with hematoxylin and eosin staining revealed a continuous cellular layer present in each of the seeded SMVs. The cellular layer appeared to be most confluent at the apex of the SMV, and there were few cells within a 5 mm zone from the SMV base, adjacent to the Dacron sewing ring. No cells were seen lining the surface of the unseeded SMV. Rather, a fibrous layer of noncellular elements was seen in the unseeded SMV.

Fluorescent microscopy. Fluorescent microscopy revealed fluorescence resulting from the PKH-26 marker in all seeded SMVs. Photographic evaluation of the video images obtained from the T8500 analyzer showed that the fluorescence was cellular in nature. In contrast, no cellular elements were noted in the unseeded SMV, only background autofluorescence from the tissues fixed in glutaraldehyde and formaldehyde.

Immunofluorescent staining with vWF antibodies also showed endothelial cells lining the SMV lumen. Histologically, the vWF-labeled fluorescent sections appeared similar to those obtained with PKH-26. A vWF-labeled segment from a representative section of an SMV surface is shown in Fig. 2. In comparing determinations of percent endothelial cell coverage of the SMV lumen from both PKH-26 and vWF, we noted a trend toward increased percentage of coverage when cells were stained with vWF. The percentage of coverage of endothelial cells for both PKH-26 fluorescence and vWF immunofluorescence is shown in Table II.

Table II. *Quantitative assessment of endothelial monolayer*

Section of SMV	*EC confluency by PKH-26 (%)*	*EC confluency by vWF (%)*
Center	52.3 ± 7.3	63.2 ± 14.7
Apex	68.4 ± 12.6	78.8 ± 8.9

EC, Endothelial cells ($n = 5$).

Scanning electron microscopy of the luminal surface of the unseeded SMV revealed a rough, fibrous surface with numerous platelets and other blood elements attached to the fibrous surface (Fig. 3). In contrast, scanning electron micrographs of the seeded SMV revealed a smooth, cellular surface with few blood elements adherent (Fig. 4). When these cells were viewed under a high-power transmission electron microscope, ultrastructural characteristics of endothelial cells such as tight intercellular junctions and Weibel-Palade bodies were apparent, helping to confirm the endothelial nature of the cells lining the SMV lumen.

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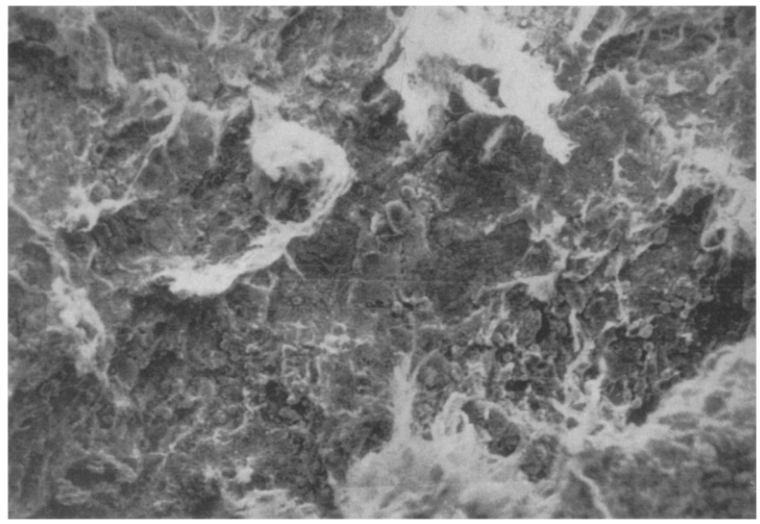

Fig. 3. Scanning electron micrograph of the luminal surface of the unseeded (control) SMV (original magnification ×500). The luminal surface is rough and multiple layers of fibers are seen along the surface. Platelets and other blood elements can be seen adherent to the SMV wall.

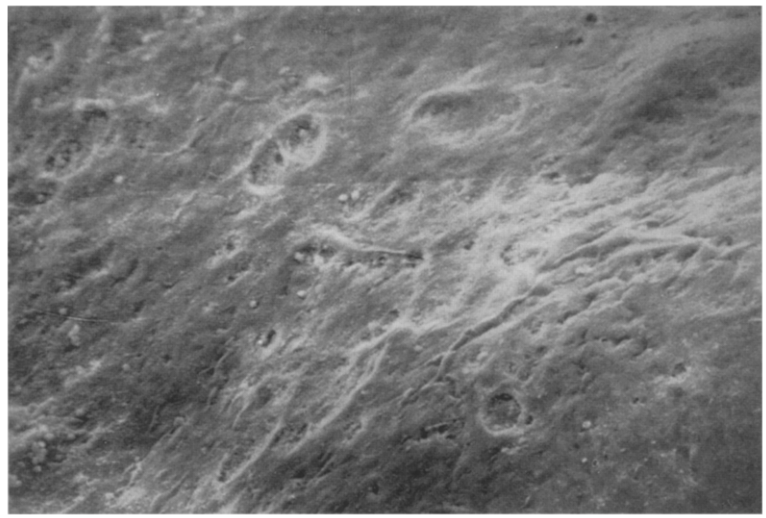

Fig. 4. Scanning electron micrograph of the luminal surface of a seeded SMV (original magnification ×470). In contrast to the seeded SMV, a smooth, cellular lining is present. Only minimal platelet deposition can be seen on the surface of the SMV.

Discussion

Over the past several years, we have worked toward developing autogenous muscle pumping chambers (SMVs) for cardiac assist. In early in-circulation studies, thrombus formation was problematic; the incidence of thrombus formation was nearly 100% and embolism occurred in 33% of animals.[11] Improvements in SMV design and configuration have improved the incidence of thrombosis but have not eliminated it altogether. In one recent study, Nakajima and associates[12] noted a 54.5% incidence of thrombus formation inside the SMV, although the incidence of embolism was only 5%.

In an effort to further decrease or eliminate the risk of SMV thrombosis and embolism, we[5, 6] have previously demonstrated the ability to successfully seed SMVs with endothelial cells using the same techniques as described herein. The clinical effectiveness of endothelial cell seeding for decreasing

the likelihood of thrombosis was first demonstrated by Herring, Gardner, and Glover[13] in 1978. They reported a threefold decrease in the thrombosis of endothelialized vascular grafts compared with unseeded control grafts. The mechanism by which endothelial cells promote an antithrombogenic environment is not fully understood but appears to be a balance of procoagulant and anticoagulant activity.[14] With regard to anticoagulant activity, endothelial cells have been shown to bind antithrombin III, locally inhibiting the coagulation cascade.[15, 16] Endothelium also inhibits platelet aggregation through prostaglandin I_2 production.[17] Factor IXa is also modulated by the endothelial cell to effect coagulant homeostasis.

The endothelial character of the cells lining the seeded SMVs was confirmed by specific stains, as well as morphologic characteristics. Hematoxylin and eosin staining demonstrates the presence of a continuous, single monolayer of cells. However, this is only suggestive for endothelial cells. The presence of PKH-26 fluorescence indicates that the cells lining the SMV lumen are either the originally seeded cells or their direct progeny. PKH-26 is an established marker that has been used to trace the fate of cells in vivo. In a rabbit model, this lipid-bound fluorescent label has been shown to remain bound to red blood cells in vitro for more than 60 days.[10] In previous studies, we[6] have shown that PKH-26 can be identified in seeded cells for at least 3 weeks in vivo, whereas in vitro the marker is traceable for more than 20 population doublings over a period of 6 weeks.

Immunofluorescent stains with antibodies to vWF have been shown to specifically bind to endothelial cells.[18] In this study, the percentage of coverage of the SMV luminal surface was greater with vWF than with PKH-26. In earlier studies, we[19] have addressed the differences seen in the number of cells containing PKH-26 and other endothelial markers, such as vWF, dil-acetylated low-density lipoprotein. Although there may be a loss of PKH-26 fluorescence, it is also possible that either the seeding process or else the presence of endothelial cells provides chemotactic or angiogenic factors inducing the growth of autogenous endothelial cells from another (nonseeded) source into the SMV lumen. In the prior study, close examination of the basement membrane level of seeded SMVs showed numerous subluminal blood vessels when compared with unseeded controls, supportive of possible ingrowth of endothelial cells from an unseeded source.[19]

One of the objectives of this current study was to determine if a monolayer of endothelial cells could be retained under in vivo shear stress conditions present in an actively contracting ventricle. In our previous studies, in which SMVs were not used to pump blood in the circulation, an 80% to 100% monolayer of cells was obtained along the luminal surface.[6] This monolayer remained intact during electrical stimulation necessary to achieve fatigue resistance. The decrease in the percent endothelial coverage of the SMV luminal surface between the previous study (80% to 100%) and this current study (50% to 80%) may be related to the effects of shear stress on the endothelium. In vivo studies of seeded vascular prostheses demonstrate significant loss of endothelial cells during pulsatile flow.[20, 21] In those studies, most of the cell loss was within the first 1 to 2 hours. Although additional cell loss may occur as a result of shear stress beyond the first 3 hours, prior studies with vascular prostheses suggest that the remaining cells would likely remain adherent.[22] The fact that the endothelial cells in this experiment were seeded onto a collagenous, fibrous surface rather than a synthetic one would likely serve to increase endothelial retention during shear stress. In fact, modification of synthetic grafts with matrix proteins such as collagen or fibronectin are used for improving endothelial retention.[23, 24]

Alternatively, the decrease in endothelial coverage may be related to mechanical trauma to the lining while the SMV is being placed in circulation. The thin, endothelial monolayer is extremely sensitive to atmospheric exposure or handling. Few cells were seen at the SMV base, within 5 mm of the sewing ring, whereas the SMV apices were invariably covered with a monolayer of cells. This observation suggests that the base may have been partially denuded of endothelial cells during the process of removing the SMV mandrel and anastomosing the SMV to the polytetrafluoroethylene graft. Although an incomplete monolayer may be thrombogenic, the possibility exists for continued growth of the apical monolayer to cover the denuded strip toward the sewing ring. Additionally, we have noted in prior studies that clot generally develops initially at the apex of the SMV, which is where the maximal confluent coverage of the SMV occurred.[12]

This study indicates that, over a 3-hour period, endothelial cell–lined SMVs can effectively function in the systemic circulation while retaining their endothelial monolayer. To our knowledge, this is the first time that an autogenous endothelial cell–

lined cardiac assist device has retained endothelium while functioning in the systemic circulation. One previous report has demonstrated that endothelial cells can be cultured from the linings of ventricular assist devices.[25] However, those findings differ from the current experiment in that the autogenous cells in this experiment were specifically seeded onto the surface to affect thrombogenicity.

The existence of an endothelialized contractile pump holds great implications for the decreasing thromboembolic events in both mechanical and skeletal muscle–powered assist devices. It remains to be seen whether these endothelialized SMVs will markedly change the current high incidence of thrombosis associated with SMVs in circulation. Resolution of thrombosis and subsequent embolism will bring SMVs nearer to eventual clinical application.

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Discussion

Mr. John H. Kennedy *(Cambridge, England).* You are to be congratulated for this novel approach to the everlasting problem of lining an artificial ventricle of one sort or another with something so that thrombosis will not occur. Could you comment on what the likely life span of your newly seeded endothelial cells would be?

Dr. Thomas. That is something we are going to have to define in further studies. We have been able to show in preliminary experiments that these cells are viable and have been present inside the SMVs for more than about a month, because the seeding process is done in the middle of the conditioning process for the SMVs.

We currently are looking at 2-week and 2-month in-circulation studies, but I cannot comment on whether we will actually find the endothelial cells inside the SMVs at that time. If we do, then we hope that this will help to decrease the problem with thrombosis that has been present in prior series.

Dr. Yasuharu Noishiki *(Yokohama, Japan).* With a vascular prosthesis, a fully endothelialized surface is rare. Most seeded endothelial cells cannot survive in vivo. But a certain number of cells are needed on the surface for these cells to survive further. For example, in case of cancer cells, more than 50 cells are required to make a new colony, namely, a new metastasis.

To enhance the endothelialization, I reported a method to accelerate endothelialization of vascular prostheses with autologous tissue fragment transplantation at the annual meeting held in Los Angeles. One of the research groups in Osaka already reported a successful result using my method in vivo, and a complete endothelialized surface was demonstrated in an experiment similar to that which you did.

Their paper was already reported in the *Japanese Society of Artificial Organs;* therefore, I am afraid that your experiment is not the first case of endothelialization.

My question is this: How high is the survival rate of the endothelial cells and what is the mechanism for the good endothelialization in your experiment?

Dr. Thomas. In this experiment we used endothelial cells that were grown in tissue culture so that we could obtain high numbers at the time of seeding. Some of the series involving the seeding of vascular grafts have obtained endothelial cells enzymatically from either the jugular vein or other sources directly at the time of seeding; those methods generally have not been able to produce a sufficient number of cells for seeding a very large area such as the inner lining of a ventricular assist device or an SMV. We have generally used between 5 million and 8 million cells grown in tissue culture for seeding purposes.

Further studies will be necessary to determine the long-term survival of these cells. We are conducting a number of preliminary studies in which we are trying to line a static or noncontracting SMV. At 2 weeks we usually have around 50% coverage of the SMV. These cells continue to grow and populate the undersurface of the SMV until at 4 to 6 weeks coverage is 100%.

We had a slight decrease in percent coverage in this series, probably because of manipulation when the SMV was being put into the circulation. As you recall, there were very few cells next to the Teflon sewing ring that we used to construct the anastomosis, whereas down at the apex there is a great confluency of cells.

Whether this trend will continue as the SMVs are in circulation for longer periods remains to be seen, but many of the kinetic studies that have shown loss of endothelial cells in vascular grafts show that the great majority are lost in the first several hours when they are first in circulation. After 24 to 48 hours this tends to stabilize and the cells can then continue to slowly grow and repopulate the surface.